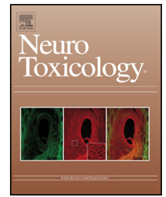
NeuroToxicology 52 (2016) 64–71

Contents lists available at ScienceDirect

NeuroToxicology



Full length article

The effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin on the development and function of the blood–brain barrier

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ARTICLE INFO

Article history:
Received 19 May 2015
Received in revised form 5 November 2015
Accepted 9 November 2015
Available online 12 November 2015

Keywords:
Blood–brain barrier
Dioxins
Brain development
Glial cell line-derived neurotrophic factor

ABSTRACT

Prenatal exposure to environmental chemicals such as dioxins is known to have adverse effects on the developing central nervous system (CNS) in mammals. Because the fetal blood–brain barrier (BBB) is immature, dioxins are thought to exert their toxic effects on the CNS by crossing the BBB and acting on neural cells directly. However, little is known whether dioxins alter the BBB. In this study, to investigate the effects of dioxins on BBB function, we exposed an in vitro BBB system comprising rat endothelial cells, astrocytes, and pericytes to the toxicant 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) either before or after BBB formation. We assessed BBB permeability and the function of tight junctions by measuring transendothelial electric resistance (TEER) values following exposure. Subsequently, total RNA and proteins were obtained from the cells for analysis. TEER values following TCDD exposure before but not after BBB formation were lower than those of the control group. We also observed that the expression of the tight junction proteins ZO-1 and claudin-5 was suppressed following TCDD exposure. To examine the cause of this reduction in protein levels, we performed a real-time quantitative polymerase chain reaction assay and observed low expression of the glial cell line-derived neurotrophic factor (GDNF) mRNA in the exposed groups. Moreover, to rescue the effects of TCDD, we applied extrinsic GDNF with TCDD. The several disruptions caused by TCDD were rescued by the GDNF addition. Our findings suggest that exposure to TCDD during BBB formation disrupts and impairs BBB function in part by the suppression of GDNF action, which may contribute to the adverse effects of TCDD on the fetal CNS.

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1. Introduction

The blood–brain barrier (BBB) is a unique system that supports and protects central nervous system (CNS) function. The circulation of blood through blood vessels in the brain is essential to deliver oxygen and nutrients to the brain and neurons and to remove metabolic waste and carbon dioxide from the brain (Daneman and Prat, 2015). The BBB tightly regulates the movement of ions, molecules, and cells using various transport systems (Ohtsuki and Terasaki, 2007). To achieve CNS homeostasis, the BBB allows nutrients and molecules to cross the BBB, while restricting the invasion of many toxicants and pathogens. The loss of this barrier function underlies serious neurological diseases such as multiple sclerosis, Alzheimer's disease, epilepsy, and brain injury (Zlokovic, 2008). Conversely, the highly selective function of the BBB prevents the delivery of CNS-acting drugs (Larsen et al., 2014).

The BBB comprises of endothelial, glial, neural, and immune cells. Endothelial cells from blood vessels resemble tubes and typically possess three transport modes, whereas astrocytes, pericytes, neural cells, and immune cells regulate and support their function (Daneman and Prat, 2015). In the rat cerebral cortex, BBB formation begins with angiogenesis on embryonic day (E) 12. Subsequently, pericytes are found around endothelial vessels. Oligodendroglial progenitor cells are evident on E19, and finally, astrocytes are detected after birth. Through these steps, the BBB is constructed, but BBB function is already present on E21, before the generation of astrocytes (Daneman et al., 2010). Astrocytes have a critical role in the development, maturation, and maintenance of the brain (Gee and Keller, 2005). BBB development and regulation are also supported by astrocytes, which produce several factors that modulate endothelial cell function.

Glial cell line-derived neurotrophic factor (GDNF) and Sonic hedgehog (Shh) are factors known to be secreted by astrocytes. GDNF is a member of the transforming growth factor-β superfamily (Lin et al., 1993). Several reports (Beck et al., 1995; Pascual et al., 2008; Tomac et al., 1995) have demonstrated that GDNF acts as a neurotrophic factor through GDNF family receptors

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http://dx.doi.org/10.1016/j.neuro.2015.11.003
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and supports neuronal functions such as neuronal cell survival, synapse formation, and cell migration. GDNF modulates the expression of the tight junction (TJ) proteins ZO-1 and claudin-5, which maintain BBB permeability (Shimizu et al., 2012). Shh binds to its receptor on endothelial cells and induces the expression of TJ proteins, including occludin and claudin-5 (Alvarez et al., 2011). This upregulation of junctional proteins increases the transendothelial electric resistance (TEER) of the BBB, thereby decreasing BBB permeability (Obermeier et al., 2013).

Many environmental chemicals with adverse effects on human CNS and brain development have been identified (Jacobson and Jacobson, 1996; Jacobson and Jacobson, 2002; Patandin et al., 1999). One such toxicant is 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), and several studies have reported its effects on the brain. Maternal exposure to TCDD distributes and accumulates the compound to maternal peripheral tissues, adipose tissue, liver, blood and breast milk by its high lipophilicity. These compounds transferred to the fetus via the placenta and lactation. Consequently, the levels of TCDD localized in the maternal liver are high, although at much lower levels, TCDD is also distributed to the fetal brain (Hurst et al., 2000; Ishida et al., 2010). Abnormal brain development and maturation may be caused by TCDD localization in neuronal cells in the fetal brain. TCDD is considered able to cross the fetal BBB to reach the brain tissue and neurons with ease because BBB function in the fetus is immature. However, it is unclear whether TCDD permeates the whole brain after crossing the BBB, and the effects of TCDD on the BBB itself are largely unknown.

In this study, we examined the effects of TCDD on BBB formation and function using an in vitro BBB model comprising three cell types: endothelial cells, pericytes, and astrocytes. As we mentioned above, BBB is composed of these cells, and the lack of any of these constituents increases BBB permeability, reduces TEER, and suppresses the expression of TJ proteins (Nakagawa et al., 2009). This system shows in vivo BBB features, and it has been utilized for drug transport assays and research on BBB pathophysiology. Although, to date, there are some reports used adult animal models or cell lines without pericytes and/or astrocytes for BBB disruption by toxicants such as TCDD and PCBs, however, no studies have analyzed the effects on BBB formation or function by exposure to TCDD or PCBs in immature BBB. We hypothesized that the adverse effects of TCDD exposure may be caused by the disruption of BBB formation and/or function. To investigate this hypothesis, we exposed the BBB system to TCDD either before or after BBB formation and investigated BBB function by measuring TEER values. Moreover, we examined changes in gene and protein expression by endothelial cells and astrocytes using a real-time quantitative reverse-transcription polymerase chain reaction (RT-qPCR) assay and immunoblotting.

2. Materials and methods

2.1. Reagents

We obtained TCDD from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA); all congeners were >98% pure. Dimethyl sulfoxide (DMSO), used as a control, was obtained from Sigma-Aldrich Co. (MO, USA). Rat recombinant glial-derived neurotrophic factor was obtained from Peprotech (Rocky Hill, NJ, USA).

2.2. The in vitro rat blood–brain barrier model

We purchased an in vitro rat BBB model system (RBT-24H BBB Kit^{TM}; PharmaCo-Cell Co. Ltd., Nagasaki, Japan). This system uses three cell types, primary cultures of rat (Wistar rat) brain capillary endothelial cells, brain pericytes and astrocytes, and the triple co-culture systems shows in vivo BBB features (Nakagawa et al., 2007; Nakagawa et al., 2009). We used it according to the manufacturer's protocol. We measured TEER to analyze the permeability of and effect of TJ on the BBB with or without exposure to TCDD. The methods used to obtain measurements were based on those used in a previous report (Nakagawa et al., 2009) and on the manufacturer's protocol. The condition of the BBB of each kit was confirmed by a TEER value of >150 ohm (Ω) × cm^2 after a 96 h pre-incubation before and after every experiment. The experimental schemes in this study are described in Fig. 1. In this study, we used two different schemes. Fig. 1A shows basic BBB formation according to the manufacturer's protocol. Four days prior to starting any assay using a mature BBB, the frozen BBB system was thawed with fresh medium provided in the kit. After thawing, the medium was changed twice, at 2–3 h and on day 1. Finally, the BBB system was constructed and was allowed to mature till day 4. First, we exposed the mature BBB to TCDD from day 4 as described in Fig. 1B, and TEER values were measured at 0, 6, 12, and 24 h after exposure. Next, we exposed the BBB kit to TCDD from day 1, at which time the BBB was immature (Fig. 1C), and measured TEER values five times at 24-h intervals (Days 1, 2, 3, 4, and 5). After the final TEER measurement, we harvested the endothelial cells and astrocytes for the real-time qPCR and immunoblotting assays from both schemas. Because we also performed the experiments to rescue the TCDD effects using extrinsic GDNF, GDNF (100 μg/μl) was applied with TCDD in the schema based on Fig. 1C.

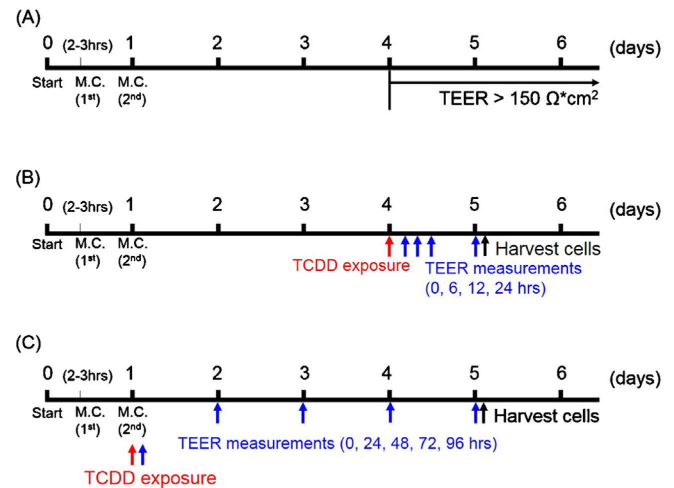

Fig. 1. Blood–brain barrier formation and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin exposure. Schema of blood–brain barrier (BBB) formation (A) according to the manufacturer of the in vitro rat BBB model system. (B) Schema of TCDD exposure on day 4 after BBB formation. (C) Schema of TCDD exposure on day 1 before BBB formation.

2.3. Real-time quantitative reverse-transcription polymerase chain reaction assay

Total RNA was isolated from astrocytes using TRIzol® Reagent (ThermoFisher Scientific, MA). Total RNA (500 ng) was reverse transcribed using ReverTra Ace® qPCR RT Master Mix (Toyobo Co., Ltd., Osaka, Japan). Quantitative real-time PCR was performed using a StepOne^{TM} Real-Time PCR System (Applied Biosystems Inc., Foster City, CA, USA) with THUNDERBIRD® SYBR® qPCR Mix (Toyobo Co., Ltd.). All real-time qRT-PCRs were performed in a 20-μl reaction mixture, according to the manufacturer's protocol, for 40 cycles using the following parameters: 95 °C for 10 min, 95 °C for 15 s, and 60 °C for 1 min. We used the following primers: CYP1A1, (F) 5′-TTCTCTTGGTTTGGGCAAG-3′, (R) 5′-GCCCATAGG-CAGGAGTCATA-3′; CYP1B1, (F) 5′-GGACAAGGACGGCTTCATTA-3′,

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